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Aging Decreases L-Type Calcium Channel Currents and Pacemaker Firing Fidelity in Substantia Nigra Dopamine Neurons

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Substantia nigra dopamine neurons are involved in behavioral processes that include cognition, reward learning, and voluntary movement. Selective deterioration of these neurons is responsible for the motor deficits associated with Parkinson's disease (PD). Aging is the leading risk factor for PD, suggesting that adaptations occurring in dopamine neurons during normal aging may predispose individuals to the development of PD. Previous studies suggest that the unique set of ion conductances that drive spontaneous, rhythmic firing of action potentials could predispose substantia nigra dopamine neurons to selective neurodegeneration. Here we show, using patch-clamp electrophysiological recordings in brain slices, that substantia nigra dopamine neurons from mice 25–30 months of age (old) have comparable membrane capacitance and input resistance to neurons from mice 2–7 months of age (young). However, neurons from old mice exhibit slower firing rates, narrower spike widths, and more variable interspike intervals compared with neurons from young mice. Dopamine neurons from old mice also exhibit smaller L-type calcium channel currents, providing a plausible mechanism that likely contributes to the changes in impulse activity. Age-related decrements in the physiological function of dopamine neurons could contribute to the decrease in voluntary movement and other dopamine-mediated behaviors observed in aging populations. Furthermore, as pharmacological antagonism of L-type calcium channels has been proposed as a potential treatment for the early stages of PD, our results could point to a limited temporal window of opportunity for this therapeutic intervention.

Key words: dopamine; firing; L-type calcium channels; mouse; pacemaking

Introduction

Dopamine (DA) neurons of the substantia nigra contribute to behaviors that include voluntary movement, sequence learning, and feeding (Hnasko et al., 2006; Jin and Costa, 2010). Mass degeneration of these neurons is a hallmark sign of Parkinson's disease (PD) and is responsible for the motor symptoms of the disease (Hornykiewicz, 1975). Aging is the leading risk factor for the development of PD, and some behavioral symptoms, including bradykinesia and cognitive decline, are common to both aging and PD. Unfortunately, little is currently known about how advancing age affects specific ion conductances responsible for DA neuron function.

DA neurons are autonomous pacemakers that fire action potentials in the absence of excitatory synaptic input (Surmeier et al., 2005). Several intrinsic conductances that are active at subthreshold potentials contribute to this tonic firing, including a persistent sodium current, L-type calcium currents, and the hyperpolarization-activated cation current I_H (Neuhoff et al., 2002; Puopolo et al., 2007; Putzier et al., 2009). Conversely, small conductance calcium-activated potassium (SK) channels and A-type potassium channels tend to slow and/or affect the regularity of pacemaker firing (Shepard and Bunney, 1991; Liss et al., 2001; Wolfart et al., 2001; Wolfart and Roeper, 2002; Hahn et al., 2003, 2006; Khaliq and Bean, 2008). DA neurons in the substantia nigra become more dependent on calcium channels to drive tonic firing as animals progress from adolescence into adulthood (Chan et al., 2007), and increased calcium handling could predispose nigral neurons to oxidative damage (Guzman et al., 2010). This has produced speculation that L-type calcium channel antagonists commonly used as antihypertensives could also be beneficial therapeutics during early stages of PD.

Little is currently known about how advancing age affects specific ion conductances responsible for DA neuron impulse activity. Because of the challenging nature of patch-clamp re-

cordings in brain slices from old mice, an electrophysiological analysis of individual conductances in DA neurons from old mice has not been reported. Measures such as transcardial perfusion of a chilled solution are sometimes used to improve the viability of neurons in slice preparations (Chen et al., 2008; Dryanovski et al., 2013), but this has not been used to study DA neurons in slices from rodents that are approaching their life expectancy. Thus, it is not known how intrinsic conductances are altered in DA neurons from extremely old animals and how this could affect tonic firing.

To address this issue, we optimized our brain slicing procedure to enable recordings of DA neurons from mice very near to their life expectancy (25–30 months) and from young adult mice (2–7 months). Remarkably, most of the electrophysiological parameters we measured were unaffected by aging. However, pacemaker firing rate and fidelity were significantly impaired in neurons from old mice. Although most intrinsic conductances that we tested were unaffected by age, we did observe dramatically smaller L-type calcium channel currents in intact neurons from old mice. Because L-type calcium channel antagonists have been posited as a possible treatment for PD, our findings could suggest a limited time window for the efficacy of this therapeutic intervention.

Materials and Methods

Brain slice electrophysiology. Young (2–7 months) and old (25–30 months) male C57BL6J mice were obtained from the National Institute on Aging aged rodent colony and group housed under a 14:10 light cycle with free access to food and water. Old mice were on average slightly larger and a subset that were measured had a mean weight of 31.8 ± 1.1 g versus 28.8 ± 0.71 g for young mice, weighed just before death ($n = 7$). On the day of recording, mice were anesthetized with 2,2,2-tribromoethanol (300–500 mg/kg) and perfused transcardially for 1 min with oxygenated, ice-cold physiological aCSF containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.4 NaH_2PO_4, 25 $NaHCO_3$, 11 D-glucose, and 1.25 kynurenic acid. Brains were extracted, blocked, and mounted on a vibrating microtome (Leica Microsystems). Horizontal slices (190–200 μm thick) of the ventral mesencephalon containing the substantia nigra were collected and thereafter maintained before recording at 34°C in aCSF plus the NMDA receptor antagonist MK-801 (10 μM), continuously bubbled with 95% O_2/5% CO_2. Recordings were performed at 34°C under continual perfusion of aCSF at a flow rate of 2 ml/min.

Putative dopamine neurons in the substantia nigra pars compacta were first identified visually as the large neurons very near (but not directly lateral to) the medial terminal nucleus of the accessory optic tract. They were further classified electrophysiologically by their spontaneous, slow firing (<5 Hz) of wide extracellular waveforms, membrane capacitance of >20 pF, input resistance in the low MΩ range, and by an I_H current >100 pA (Ford et al., 2006; Branch and Beckstead, 2012). Pipettes of 9–10 MΩ resistance filled with a Na HEPES-based solution (plus 20 mM NaCl, 290 mOsm/L, pH 7.40) were used for extracellular recordings using the loose cell attached method as previously described (Branch and Beckstead, 2012). Whole-cell patch recordings were made with 1.8–2.0 MΩ resistance pipettes filled with internal solution (in mM) as follows: 115 K-methylsulfate or cesium chloride, 20 NaCl, 1.5 $MgCl_2$, 10 HEPES, 0.025 EGTA, 2 ATP, and 0.4 GTP, pH 7.35–7.40, 267–275 mOsm/L. The experiments described in Figure 4 included the addition of neurotransmitter receptor antagonists to the aCSF: picrotoxin (100 μM, $GABA_A$), CGP55845 (100 nM, $GABA_B$), DNQX (10 μM, AMPA), and hexamethonium (100 μM, nACh-R). Blockers used at various times to isolate ionic conductances included apamin (100 nM, SK channels), tetrodotoxin (TTX, 500 nM, voltage-gated Na channels), tetraethylammonium (TEA, 1 mM, K channels), and nimodipine (3 μM, L-type Ca channels). Liquid junction potentials were measured and corrected using the method described by Neher (1992).

Data were acquired at 50 kHz and filtered at 6 kHz using a Multiclamp 700B amplifier (Molecular Devices). Data were recorded and later analyzed offline using Axograph X software (www.axograph.com). Voltage ramps were resampled at 0.014 kHz (1 mV increments) for analysis.

RNA extraction and real-time quantitative PCR. Dissected substantia nigra from young ($n = 7$) and old ($n = 4$ or 5) mice were stored in RNA*later* (QIAGEN) at −80°C. After homogenization using TissueMiser (Fisher Scientific), RNA was extracted using RNeasy mini Plus kit (QIAGEN) as per the manufacturer's instructions. Concentration was quantified (Nanodrop Products) and sample quality verified based on 260/280 ratio. RNA was converted to cDNA using qScript (Quanta Biosciences). Expression levels were analyzed on an AB 7900 Real Time PCR System (Invitrogen) using SYBR Select Master Mix (Invitrogen). Cycle conditions were as follows: 95°C for 15 s, 60°C for 1 min. Target gene expression levels were normalized to expression of the endogenous reference control gene, $eEF1\alpha1$. All reactions were performed in triplicates with standard curves for each primer set and analyzed using the Relative Expression Software Tool (Pfaffl et al., 2002). The presence of single PCR products was confirmed based on dissociation curves and by separation on agarose gels (data not shown). PCR was performed using the following primers (forward followed by reverse): *Scn1a*: 5′-TCAGAGGGAAGCACAGTAGAC-3′ and 5′-TTC-CACGCTGATTTGACAGCA-3′; *Scn2a*: 5′-ATTTTCGGCTCATTCTTCA-CACT-3′ and 5′-GGGCGAGGTATCGGTTTTTGT-3′; *Scn5a*: 5′-AT GGCAAACTTCCTGTTACCTC-3′ and 5′-CCACGGGCTTGTTTTTCAGC-3′; *Scn1b*: 5′-GGATTCCGATACCGAGGCTG-3′ and 5′-GGTCCACTCCGT-GAAGGTTT-3′; *Scn2b*: 5′-AGTAAGTACGACGTGTCAGTGA-3′ and 5′-AGGGTTGGTAATGTAGCAGTTG-3′; *Scn3b*: 5′-ATGTGTCCAGG GAGTTTGAGT-3′ and 5′-TTCGGCCTTAGAGACCTTTCT-3′; *Hcn1*: 5′-CAAATTCTCCCTCCGCATGTT-3′ and 5′-TGAAGAACGTGATTC-CAACTGG-3′; *Kcnn3*: 5′-TGTTGCACTCTTCTCCCACG-3′ and 5′-GGTCATTGAGATTTAGCTGGCT-3′; *Cacna1c*: 5′-ATGAAAACA CGAGGATGTACGTT-3′ and 5′-ACTGACGGTAGAGATGGTTGC-3′; *Cacna1d*: 5′-AGAGGACCATGCGAACGAG-3′ and 5′-CCTTCACCA-GAAATAGGGAGTCT-3′; *eEf1α1*: 5′-ATTCCGGCAAGTCCACCACAA-3′ and 5′-CATCTCAGCAGCCTCCTTCTCAAAC-3′.

Immunohistochemistry. Dopamine cells of the substantia nigra were patch-clamped with pipettes (2 MΩ) filled with an internal solution containing 0.2% neurobiotin (Vector Laboratories). After electrophysiological recordings, brain slices were postfixed in 4% PFA overnight and then transferred to a KPBS solution until processed. Initially slices were bathed in a 1% H_2O_2 solution for 15 min, washed and blocked with 5% goat serum and 0.3% Triton X-100 (Sigma-Aldrich) for 1 h. After washing with KPBS, slices were then incubated for at least 48 h in anti-tyrosine hydroxylase (final dilution 1:250; EMD Millipore) at 4°C. After washing with KPBS, the slices were exposed to the goat anti-rabbit AlexaFluor-594 (final dilution 1:750; Abcam) for 2 h. A conjugated streptavidin AlexaFluor-488 antibody (final dilution 1:750; Jackson ImmunoResearch Laboratories) was added to the secondary antibody solution for an additional hour. After sufficient washes in K-phosphate buffered saline, the slices were mounted on gelatin-coated slides and the slides were coverslipped using Prolong Gold antifade reagent (Invitrogen). Images were collected on an Olympus DP72 microscope using Olympus standard cellSens software.

Drugs. DNQX, picrotoxin, MK-801, kynurenic acid, hexamethonium chloride, guanosine 5′ triphosphate sodium salt hydrate (GTP), adenosine 5′ triphosphate magnesium salt (ATP), EGTA, cesium chloride, TTX, TEA, 2,2,2-tribromoethanol, cadmium chloride, and goat serum were purchased from Sigma-Aldrich. CGP 55845 hydrochloride and nimodipine were purchased from Tocris Bioscience. Apamin was obtained from Fisher Scientific. Methylsulfuric acid potassium salt was purchased from Acros Organics. Neurobiotin tracer was purchased from Vector Laboratories.

Data analysis. All values reported are mean ± SEM. All statistics were computed using Prism 6.0 software. Two-tailed Student's t tests and two-way ANOVAs were used to analyze data, using repeated-measures designs wherever feasible. The nonparametric Kolmogorov–Smirnov test was used to compare cumulative distributions in situations where two groups being compared had unequal variances or were not normally distributed. Linear regression was used to analyze the relationship be-
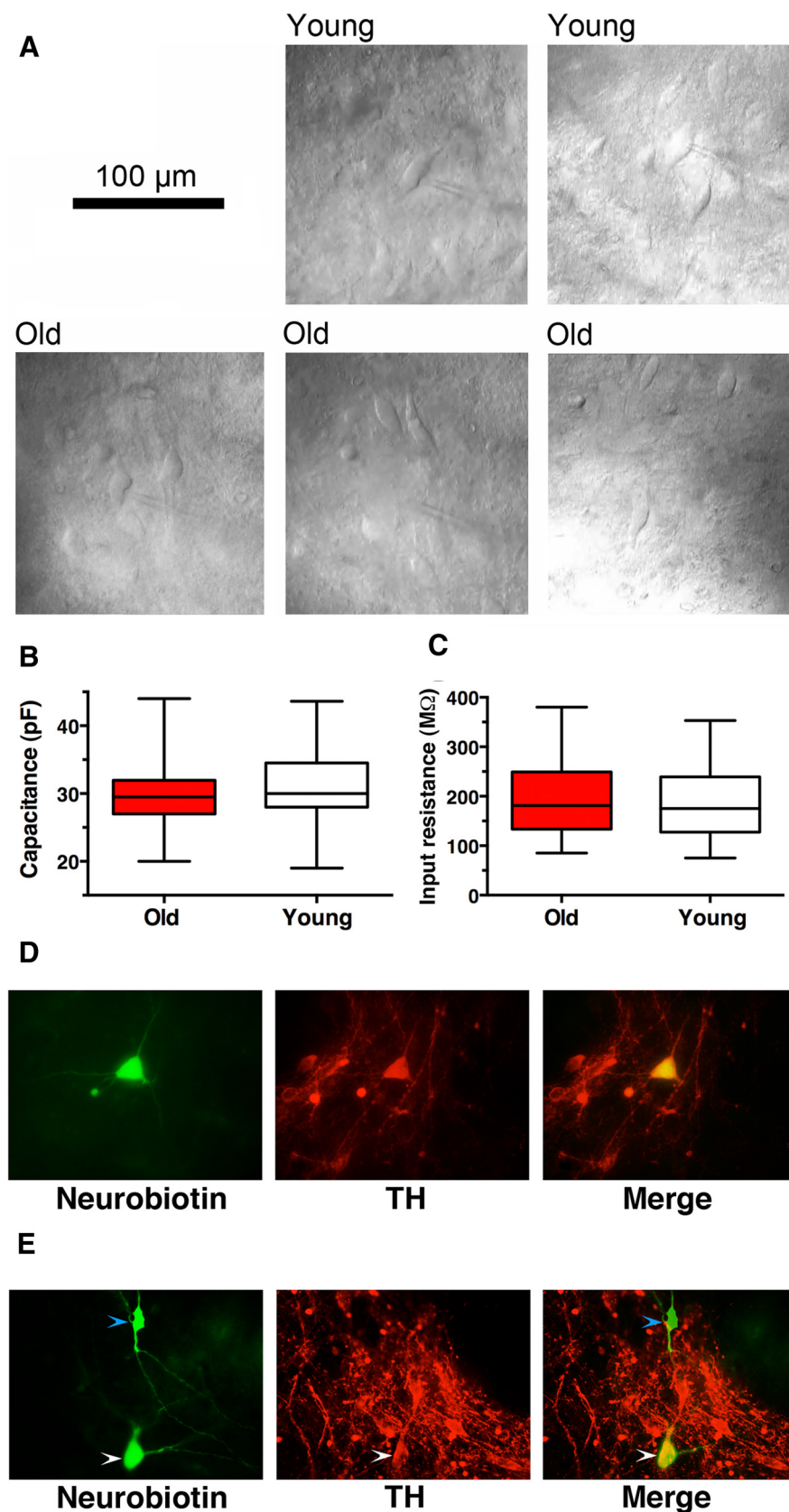
Figure 1. DA neurons from aging mice maintain their appearance and basal electrophysiological parameters. Mouse brain slices from old and young mice were examined with an upright microscope using gradient contrast optics, and DA neurons of the substantia nigra pars compacta were initially identified by location and appearance. ***A***, Brain slices from old mice (25–30 months) showed visually intact DA neurons similar to young adult (2–7 months) mice. ***B***, Cell membrane capacitance was measured and was not different in neurons from old mice compared with their young counterparts. ***C***, Input resistance was also similar in DA

tween mouse age (in weeks) and nimodipine-sensitive currents. For all analyses, α was set *a priori* at 0.05.

Results

To maximize the quality and health of the neurons in our brain slice preparation, we deeply anesthetized mice with 2,2,2-tribromoethanol (300–500 mg/kg, i.p.) and transcardially perfused the mice for 60 s with ice-cold aCSF that included the glutamate receptor antagonist kynurenic acid (1.25 mM). We then rapidly removed the brain and isolated horizontal slices of the ventral mesencephalon containing the substantia nigra. Visualization of DA neurons in slices from young adult (2–7 months) and very old (25–30 months) mice was accomplished using gradient contrast infrared optics (Dodt et al., 2002) and is depicted in Figure 1A. Although, in general, the number of visibly healthy neurons was lower in preparations from older mice, intact DA neurons with characteristic large cell bodies were visible in every preparation near the medial terminal nucleus of the accessory optic tract, regardless of the age of the mouse. Surprisingly, whole-cell recordings made in these neurons indicated that neither membrane capacitance (Kolmogorov–Smirnov test, $p = 0.31$) nor input resistance ($t_{(84)} = 0.6658$, $p = 0.51$) significantly differed between age groups (old: 29.78 ± 0.67 pF, 191.9 ± 11.6 MΩ, $n = 41$; young: 30.98 ± 0.88 pF, 181.5 ± 10.4 MΩ, $n = 45$; Fig. 1 B,C). In a subset of recordings, neurons were filled with 0.2% neurobiotin tracer, which was included in the internal solution of the recording pipette. After recordings, slices were preserved in 4% PFA and later stained for neurobiotin and for tyrosine hydroxylase (TH) to confirm their identity as dopamine neurons (Fig. 1D; $n = 23$). Because of the duration of our experiments, which often exceeded 40 min, dual staining was not observed in 10 of 33 cases. In each of these cases, an adjacent cell had also been patch-clamped for

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neurons from old and young mice. Boxplot whiskers indicate maximum and minimum data points. ***D***, In a subset of experiments, 0.2% neurobiotin was included in the intracellular solution and slices were fixed with PFA for immunohistochemistry; 70% of neurons costained for neurobiotin and TH, an indicator of catecholaminergic neurons. When whole-cell patch-clamp experiments exceeded 15 min, a nearby neuron was also briefly patch-clamped to account for the fact that false negatives occur when TH staining is attempted after long recordings (***E***, blue arrowheads) (Zhang et al., 2010); and in 9 of 10 remaining cases, the nearby neuron dual-stained for neurobiotin and TH (***E***, white arrowheads).
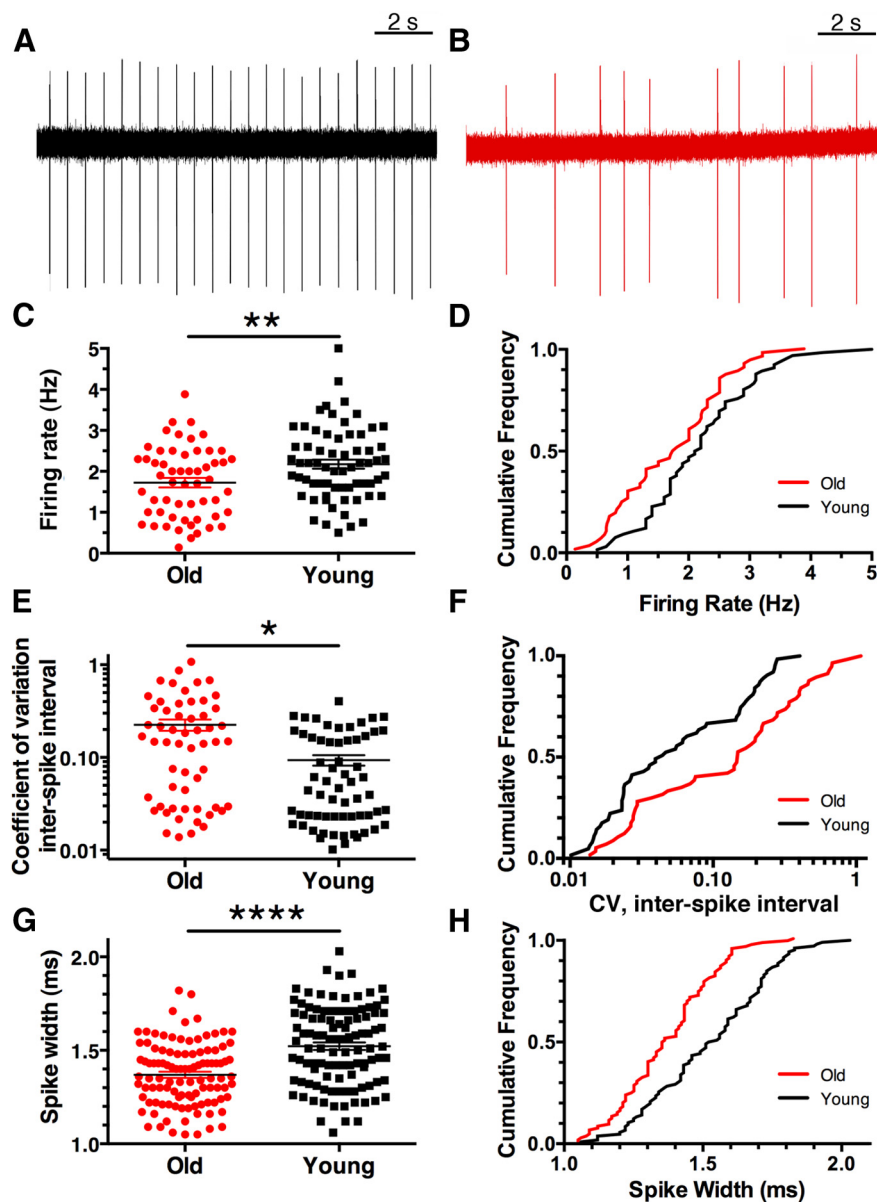
Figure 2. Pacemaker firing fidelity is compromised in DA neurons from aging mice. Intrinsic firing of substantia nigra DA neurons was monitored using loose cell attached extracellular recordings. **A**, The majority of DA neurons from young mice fired in a characteristic rhythmic pattern. **B**, In contrast, DA neurons from old mice (in red) often exhibited more varied firing that, in extreme cases, included gaps of several seconds with no spikes. **C**, **D**, Overall firing rates of neurons from old mice were slower than their young counterparts. Interspike intervals were calculated over 30 s of firing, and neurons from old mice exhibited significantly larger coefficients of variation of the interspike interval compared with neurons from young mice, indicative of a more variable firing pattern (**E**). **F**, Cumulative frequency analysis revealed a bimodal distribution for the coefficient of variation, with values <0.10 generally indicative of pacemaker firing. **G**, **H**, The kinetics of the extracellular waveforms were also analyzed and indicated that DA neurons from old mice exhibit narrower spike widths compared with their young counterparts. *$p < 0.05$. **$p < 0.01$. ****$p < 0.0001$.

30–60 s, which is sufficiently short to prevent "washout" of TH signal that is associated with recordings longer than 15 min (Zhang et al., 2010). In these cases, the nearby cell was dual-stained in 9 of 10 cases (Fig. 1E; $n = 9$), TH-positive cells were observed nearby, and in only one instance of 33 was the neuron TH- and neurobiotin-negative due to either poor slice handling and/or imaging. This indicates that DA neurons from aging mice not only maintain their appearance but also basal parameters, including their cell size and composite membrane conductance.

Substantia nigra DA neurons fire in a rhythmic pacemaker-like pattern in *ex vivo* brain slice preparations (Grace and Onn, 1989). We thus obtained loose cell-attached recordings of DA neurons to evaluate the effects of aging on pacemaker firing. Most neurons from young mice fired spikes in a characteristic rhythmic pattern (Fig. 2A). However, DA neurons from aging mice were much more likely to exhibit a disrupted spontaneous firing pattern (Fig. 2B). Mean firing rates were significantly slower in neurons from old mice (1.72 ± 0.12, $n = 56$) compared with young mice (2.18 ± 0.11, $n = 66$, $t_{(120)} = 2.831$, $p = 0.0054$; Fig. 2C,D). Spontaneous firing was recorded for 30 s, and the interspike intervals (i.e., the time between spikes) were calculated for all neurons that fired more than two action potentials during that period. The coefficient of variation of the interspike interval was significantly higher in DA neurons from old mice (0.225 ± 0.031, $n = 57$) than from their young counterparts (0.0936 ± 0.0119, $n = 63$; Kolmogorov–Smirnov test, $p = 0.016$; Fig. 2E,F). A closer evaluation of the extracellular spike waveforms was performed, and spike width was defined (in voltage-clamp mode) as the time from the initiation of the inward current to the peak of the outward current (Ford et al., 2006). Neurons from old mice exhibited a narrower spike width (1.37 ± 0.016, $n = 105$) than those from young mice (1.52 ± 0.020, $n = 106$; Kolmogorov–Smirnov test, $p < 0.0001$; Fig. 2G,H). Therefore, although aging does not affect the appearance or basal electrical properties of DA neurons, it does slow spontaneous firing rates, decrease the fidelity of pacemaker firing, and shorten spike widths.

Spontaneous firing of DA neurons in brain slice preparations is almost exclusively driven by slow intrinsic conductances. Both small-conductance Ca^{2+}-sensitive K^+ (SK) channels and hyperpolarization-activated HCN channels are major determinants of firing rate (Shepard and Bunney, 1991; Wolfart et al., 2001; Neuhoff et al., 2002). We next used whole-cell recordings to investigate the effects of aging on the function of these channels. SK channels are responsible for a slow afterhyperpolarization current (AHC) in DA neurons. We applied a 55 mV depolarizing voltage step for 100 ms, then measured the AHC as the integral of the apamin-sensitive portion of the outward current to determine the total ionic charge that was passed (Ahn et al., 2010) (Fig. 3A). We observed no difference in AHCs in DA neurons from old (42.09 ± 4.46 pA $\times$ s, $n = 22$) versus young mice (47.32 ± 5.37 pA $\times$ s, $n = 20$; $t_{(40)} = 0.755$, $p = 0.45$; Fig. 3B). We then used a 50 mV hyperpolarizing step to activate the HCN channel-mediated current I_H, which produces a

prominent nonselective cation conductance in DA neurons of the substantia nigra (Fig. 3C). There was no difference in mean I_H current in DA neurons from old (439.6 ± 21.2 pA, $n = 92$) versus young mice (456.6 ± 25.4 pA, $n = 88$; $t_{(178)} = 0.517$, $p = 0.61$; Fig. 3D). These results suggest that age-related alterations in dopamine neuron excitability likely cannot be attributed to changes in SK or HCN channel conductances.

We next examined voltage-gated sodium and calcium channel-mediated currents, both of which are also major contributors to substantia nigra DA neuron excitability (Wilson and Callaway, 2000; Puopolo et al., 2007). To examine their activity at near-threshold voltages, we performed slow (14 mV/s), depolarizing voltage ramps using a cesium-based pipette solution and extracellular TEA to minimize the contribution of potassium channels (Fig. 4A). We then bath perfused the sodium channel blocker TTX (500 nM) to determine the relative contribution of voltage-gated sodium channels in old and young mice. We observed that TTX-sensitive currents were similar in DA neurons from old and young mice
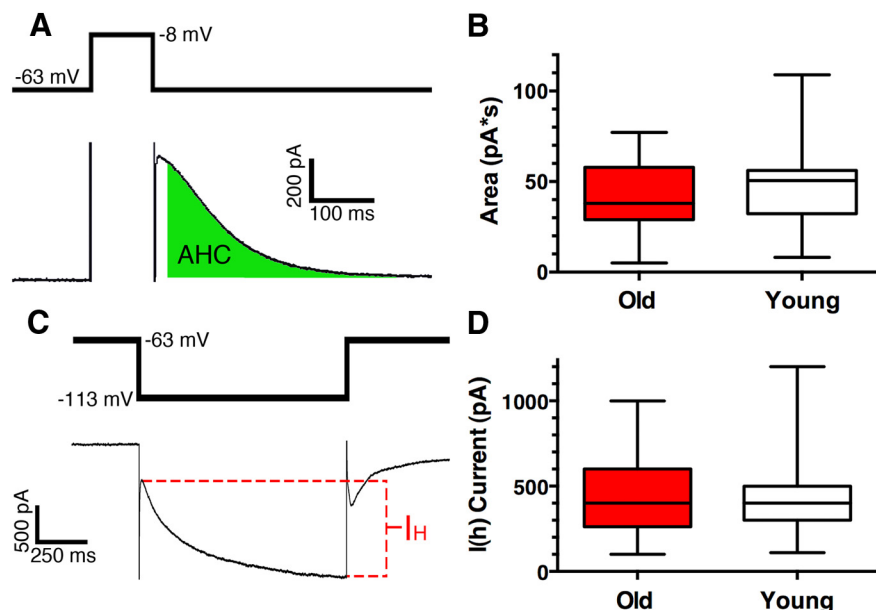

Figure 3. Currents associated with HCN and SK channels are not altered in aging mice. **A**, The AHC, which is primarily mediated by SK channels, was calculated as the area under the curve of the outward current beginning 20 ms after a 100 ms depolarization (green shading). **B**, Summary data indicate that AHCs in DA neurons did not differ with the age of the mice. **C**, I_H currents were calculated as the amplitude of the slow inward current that develops during a 1 s hyperpolarization step. **D**, Summary data show that I_H currents in neurons from old mice were similar to currents in neurons from young mice. Boxplot whiskers indicate maximum and minimum data points.

($n = 12$ and 16 cells, respectively; two-way ANOVA, main effect of age, $F_{(1,26)} = 0.1545$, $p = 0.6975$; age × voltage interaction, $F_{(56,1456)} = 0.8148$, $p = 0.8339$; Fig. 4B), indicating that age-related changes in firing patterns are not due to a difference in voltage-gated sodium conductances. With potassium and sodium channels pharmacologically blocked, we next tested the effects of the L-type calcium channel blocker nimodipine (3 μM). At near-threshold voltages, nimodipine significantly attenuated the inward current in neurons from young animals ($n = 21$; Fig. 4C), but summary data indicate that this effect was substantially smaller in neurons from old animals ($n = 26$; two-way ANOVA, main effect of age, $F_{(1,45)} = 24.1$, $p < 0.0001$; age × voltage interaction, $F_{(60,2700)} = 4.985$, $p < 0.0001$; Fig. 4D). Linear regression analysis of individual data points (Fig. 4E) indicated a significant inverse relationship between the age of the mice in weeks and the maximum amplitude of nimodipine-sensitive inward currents ($F_{(1,45)} = 11.05$, $p = 0.0018$).

Although calcium influx depolarizes the neuron, it can also activate calcium-sensitive SK channels, causing the extrusion of potassium and slowing firing rate. However, mechanistic changes of voltage-dependent calcium channels due to ages beyond young adulthood have yet to be elucidated. In an attempt to determine the contribution of L-type calcium channels to firing parameters, we obtained extracellular recordings and determined the effect of nimodipine on firing rates in pacemaking DA neurons from old and young mice. Nimodipine initially increased firing rate in neurons from young mice, followed by a slowing of firing. Neurons from old mice fired more slowly after several minutes but lacked the initial increase in firing. Repeated-measures ANOVA indicated a significant main effect of age ($n = 8$ cells/group; $F_{(1,264)} = 25.96$, $p < 0.0001$; Fig. 4F) and time ($F_{(18,264)} = 8.562$, $p < 0.0001$) but no significant age × time interaction ($F_{(18,264)} = 0.6454$, $p = 0.8618$). We also examined these neurons for nimodipine-induced changes in the coefficient of variation of the interspike interval (Fig. 4G,H). Nimodipine produced an increase in this parameter ($F_{(1,19)} = 5.436$, $p = 0.03$), but there was no significant age × nimodipine interaction ($F_{(1,19)} = 2.828$, $p = 0.11$). One caveat to this finding is that, to perform the experiment in Fig. 4F, we could only use neurons in both groups that were stably pacemaking, thus potentially skewing our data toward young-seeming neurons in slices from old mice. Furthermore, many cells in both groups ceased firing altogether during washout (data not shown), possibly indicating slow tissue penetration for nimodipine. Regardless, our data indicate that age diminishes L-type calcium channel-mediated currents in DA neurons and also alters the effect of nimodipine on pacemaker firing. These results suggest that DA neurons from young mice have a larger participation of voltage-gated calcium channels at near-threshold voltages than cells from very old mice.

Finally, we attempted to ascertain whether alterations in ion channel gene expression could be responsible for the age-related decline in physiological parameters. We isolated RNA from the substantia nigra of young and old mice, performed quantitative real time RT-PCR on 10 ion channel genes, and quantified expression by comparing it with the control reference gene $eEF1\alpha1$ (Sharma et al., 2010). These analyses revealed no significant age-related changes in gene expression (Table 1). Interestingly, the only two potential trends in these data were for increased expression in old mice of the genes that code for Cav1.2 and Cav1.3. This is in the opposite direction of our functional findings and could suggest an increase in transcription as a compensation for age-related decline in pacemaker firing. A very similar disparity between age-related changes in L-type channel expression and function has been seen in rat hippocampus, where overall Cav1.2 and Cav1.3 channel expression is decreased but channel surface expression is actually increased, matching previously observed increases in channel activity (Núñez-Santana et al., 2014).
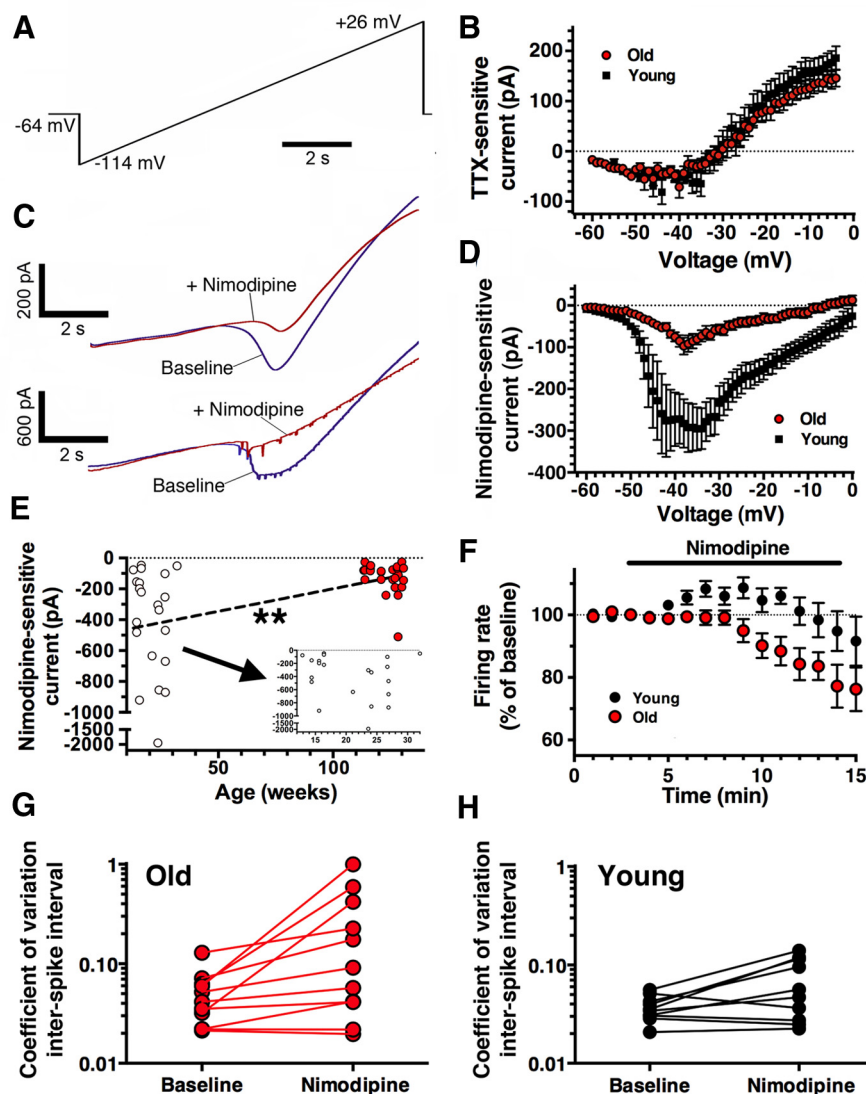
Figure 4. L-type calcium channel-mediated currents are smaller in DA neurons from aging mice. **A**, Using a cesium-based intracellular solution, whole-cell patch-clamp recordings of DA neurons were obtained and slow depolarizing voltage ramps were applied over 10 s. **B**, Currents subtracted after bath perfusion of TTX indicated that there was no effect of age on voltage-gated sodium channel-mediated currents. **C**, Two sample traces of DA neurons from young mice illustrate a nimodipine-sensitive inward current. A subset of cells exhibited brief inward current deflections in the presence of TTX that were not blocked by nimodipine (e.g., bottom trace). **D**, Summarized data indicate a dramatic decrease in the amplitude of near-threshold nimodipine-sensitive inward currents in neurons from old versus young mice. **E**, Linear regression analysis indicated a significant inverse relationship between the age of the mouse and the maximum nimodipine-sensitive currents (dashed line; $p = 0.0018$). Linear regression analysis of only the data points from young mice indicated no significant difference in the nimodipine-sensitive currents within that subset of ages (inset, $F_{(1,19)} = 0.87$, $p = 0.36$). Using the cell attached method, firing rates of neurons from old and young mice were examined before and after bath application of 3 μM nimodipine. **F**, Summarized data indicate a differential effect of nimodipine on firing rate in DA neurons from old and young mice ($p < 0.0001$). Pacemaking neurons from both old (**G**) and young (**H**) mice exhibited similar nimodipine-induced increases in the coefficient of variation of their interspike intervals.

Discussion

Here we show that DA neurons from old mice exhibit slower firing rates, impaired firing fidelity, shorter spike widths, and decreased L-type calcium currents compared with their young counterparts. We did not observe an effect of age on other electrophysiologically measured parameters, including cell size, input resistance, I_H, and SK channel currents. These findings extend our knowledge of the effects of aging on DA neuron physiology to very old time points at which neurological diseases and bradykinesia are common. This information could prove invaluable for developing pharmacological strategies to treat age-

associated brain disorders affecting the dopaminergic system. Further, because dihydropyridines have been proposed as a potential treatment for PD, our results could suggest a limited temporal window for the efficacy of this therapeutic intervention.

Intrinsic ion conductances and dopamine neuron firing

DA neurons are autonomous pacemakers that fire action potentials even in the absence of active excitatory synaptic input. *In vivo*, tonic firing of substantia nigra dopamine neurons promotes DA release in the dorsal striatum that is critical for the initiation of voluntary movement (Carli et al., 1985; Tepper et al., 1991). In *ex vivo* brain slice preparations, such as the one used presently, DA neurons typically fire in a rhythmic, pacemaker-like pattern (Grace and Onn, 1989). DA neuron pacemaking is influenced by intrinsic ion conductances, and the role of voltage-dependent calcium channels on this firing has been studied in a variety of preparations. Previous work suggests that L-type calcium currents are heavily involved in control of spontaneous firing frequency, specifically by inducing a near-threshold depolarization and a subsequent activation of an SK channel-mediated afterhyperpolarization potential (Puopolo et al., 2007). Although L-type calcium currents are generally considered to be high-voltage activated, L-type currents in substantia nigra DA neurons are largely mediated by $Ca_V1.3$ channel subunits (Takada et al., 2001), which activate at relatively negative potentials and inactivate slowly (Kang and Kitai, 1993; Wilson and Callaway, 2000). These channels drive pacemaker firing by producing slow voltage oscillations in both somatic and dendritic compartments (Grace, 1991; Kang and Kitai, 1993; Wilson and Callaway, 2000). Because pacemaker firing in the substantia nigra is dependent on near-threshold calcium currents, a decrease in channel density alone could disrupt the coupling between the dendrites and the soma and decrease firing regularity (Wilson and Callaway, 2000). The dramatically decreased nimodipine-sensitive currents that we observed in DA neurons from older mice thus likely contributes to the decreased fidelity in pacemaker firing. However, nimodipine did increase the coefficient of variation of the interspike interval in neurons from both young and old mice, indicating that age-related adaptations in other ion channels and/or neuronal structure must also be contributing factors. Calcium entering through L-type calcium channels is also a major contributor to the "shoulder" of the action potential depolarization that determines spike width (Puopolo et al., 2007). Thus, the decrease in L-type cur-

Table 1. Ion channel gene expression in young and old mice[a]

Gene	Subunit	Old	Young	p
Scn1a	Nav1.1	2.21 ± 0.24 (5)	2.17 ± 0.28 (7)	0.91
Scn2a	Nav1.2	0.88 ± 0.29 (4)	0.84 ± 0.17 (7)	0.91
Scn5a	Nav1.5	0.15 ± 0.05 (4)	0.16 ± 0.03 (7)	0.96
Scn1b	NavB1	0.89 ± 0.17 (4)	0.89 ± 0.05 (7)	0.99
Scn2b	NavB2	1.23 ± 0.26 (4)	1.34 ± 0.13 (7)	0.69
Scn3b	NavB3	1.28 ± 0.18 (4)	1.24 ± 0.08 (7)	0.82
Hcn1	HCN1	2.12 ± 0.44 (4)	1.85 ± 0.36 (7)	0.65
Kcnn3	SK3	0.24 ± 0.05 (4)	0.24 ± 0.03 (7)	0.97
Cacna1c	Cav1.2	1.32 ± 0.33 (5)	0.96 ± 0.13 (7)	0.29
Cacna1d	Cav1.3	1.10 ± 0.22 (5)	0.78 ± 0.21 (7)	0.15

[a]We performed real-time qRT-PCR on substantia nigra samples taken from young and old mice. Values are mean ± SEM of the ratio of each gene's expression in relation to the control reference gene eEF1α1, with sample size in parentheses. p values were obtained from two-tailed, unpaired t tests.

rents is consistent with the decreased spike width we observed in neurons from aging mice.

One important caveat is that, in addition to blocking Ca_V 1.2 and 1.3, 3 µM nimodipine can partially block some T-type calcium channels (Ca_V 3.2 or 3.3) (Furukawa et al., 2009). T-type channel-mediated currents have been observed in substantia nigra dopamine neurons from very young rodents and can either contribute to irregular firing (in P6-P12 rats) (Cui et al., 2004) or, in some neurons from P10-P14 mice, pacemaker regularity by activating SK channels (Wolfart and Roeper, 2002). Although we cannot currently rule out a role for T-type channels in the age-related effects of DA neuron physiology, our experimental protocols suggest that they are not responsible for the observed effects of nimodipine. We used extremely slow ramp protocols (14 mV/s) that limit the contribution of rapidly inactivating channels, such as T-type calcium channels. Furthermore, T-type activation occurs at low voltages and in DA neurons inactivates at −40 mV (Cui et al., 2004), suggesting that T-type channels are not central to the effects of nimodipine that peak positive of −40 mV. Although we did not study them in detail, the brief inward currents we observed in some cells (e.g., Fig. 4C, bottom) could have been the result of T-type channels and were not blocked by nimodipine. Finally, tissue penetration issues often necessitate perfusion of higher concentrations of antagonists in brain slices over cellular preparations (such as the Furukawa et al. 2009 study, which was performed in *Xenopus* oocytes) to produce the same effects. We believe this to be important in the present study due to the following: (1) because of the difficulty of recording from old mice, we intentionally recorded from deep cells in both groups to maximize neuronal health; (2) bath perfusion of 300 nM nimodipine (which should have blocked Ca_V 1.2 and 1.3) had very little effect on firing rate, firing fidelity, and ramp-induced currents (data not shown); and (3) 3 µM nimodipine caused firing to completely cease in many neurons from both groups during washout, suggesting that the drug was still penetrating the slice tissue. More work will be required to address this complex issue as more selective blockers of L- and T-type channel subunits become available. It is perhaps noteworthy that microdialysis data do not support a role for T-type channels in the somatodendritic release of dopamine in the substantia nigra (Bergquist and Nissbrandt, 2003).

A number of dopamine-mediated behaviors decline with age, including voluntary movement, cognition, and attention (Gerhardt et al., 2002; Nieoullon, 2002; Colebrooke et al., 2006; Gispert et al., 2009). Wholesale death of DA neurons during normal aging could explain these observations, but its actual prevalence is controversial (Kubis et al., 2000; Haycock et al., 2003). If

DA neuron death does occur with normal aging, it is only in the range of 10%–30% and alone is unlikely to explain the neurochemical and behavior deficits observed with advancing age (McGeer et al., 1977; Bäckman and Farde, 2001). Neurochemical experiments suggest that extracellular levels of DA decrease with age, perhaps by 60% in the striatum, despite an apparent decrease in DA uptake (Hebert and Gerhardt, 1998; Gerhardt et al., 2002; Haycock et al., 2003; Colebrooke et al., 2006). This suggests that, during aging, at least a portion of the surviving DA neurons develop changes in their physiology that produce a degree of functional senescence. An age-related decrease in the DA synthesizing enzyme dopa decarboxylase could contribute to this effect (Cruz-Muros et al., 2007). Our results suggest that the decreasing amplitude of the L-type calcium currents that contribute to DA neuron firing could play a role in age-related decrements in dopamine-mediated signaling and its behavioral consequences.

L-type calcium channels and dopamine neuron degeneration

The reliance on subthreshold calcium currents for pacemaker firing could explain why DA neurons in the substantia nigra are susceptible to the significant neurodegeneration required for the appearance of motor symptoms in PD (Hornykiewicz, 1975; McGeer et al., 1988; Khaliq and Bean, 2010). Increased intracellular calcium can stress the organelles responsible for maintaining calcium homeostasis and has been linked to apoptosis and the formation of reactive oxygen species (Orrenius et al., 2003). This, combined with the fact that these neurons have only a modest ability to buffer calcium (Foehring et al., 2009), suggests that any insult that increases the cytoplasmic calcium burden could expose the cells to calcium-dependent pathology. It has thus been proposed that dihydropyridines, such as nimodipine and isradipine, already in use as therapeutic agents to treat hypertension, could also protect patients from the neurodegeneration that is characteristic of PD and other age-related neuronal phenotypes. Consistent with this notion, calcium-channel blocker usage in humans decreases the chance of being diagnosed with PD (Becker et al., 2008), and isradipine administration reduces oxidative damage to DA neurons in a mouse model of PD (Ilijic et al., 2011).

The present study identified alterations in DA neuron physiology associated with normal aging and did not investigate a model of PD. However, because aging is the leading risk factor for the development of PD, our results could have important implications for the treatment of PD patients. The decline in L-type calcium currents we observed in neurons from aging (yet visually healthy) animals suggests that there may be a limited time window for treatment with L-type antagonists, likely before overt motor impairments are evident. It is also conceivable that the age-related alterations we observed in dopamine neuron physiology are part of an adaptive process designed to extend the lifespan of individual neurons by reducing L-type calcium currents, sacrificing firing fidelity in the process. Fortunately, the late onset of motor symptoms in PD suggests a relatively slow time course for DA neuron degeneration. An ideal therapeutic regimen for PD would combine symptomatic treatment with a neurorestorative intervention to protect or enhance the function of remaining neurons (Dawson and Dawson, 2002). Thus, it is key that we continue to identify adaptive changes that occur with aging and during the early stages of PD to establish improved drug therapies to halt, slow, or reverse neurodegeneration. Additionally, a carefully constructed time course of the degenerative changes occurring during disease development will be required to best determine effective therapeutic interventions.

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